

**MODERATELY LOWER TEMPERATURES GREATLY EXTEND
THE LIFESPAN OF *BRACHIONUS MANJAVACAS* (ROTIFERA):
THERMODYNAMICS OR GENE REGULATION?**

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THERMODYNAMICS OR GENE REGULATION?**

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To Alex for being my rock and keeping me away from the zero.
And to my family – thank you for being the craziest, most adventurous, and most
supportive people I know.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
SUMMARY	ix
<u>CHAPTER</u>	
1 CHAPTER 1 INTRODUCTION	1
2 CHAPTER 2 MATERIALS AND METHODS	4
Rotifer Culture	4
Experimental Design and Treatments	4
Stressor Challenge Experiments	5
Synthesizing and Purifying dsRNA	6
RNAi Knockdown Experiments	7
Statistics	8
3 CHAPTER 3 RESULTS	9
4 CHAPTER 4 DISCUSSION	19
REFERENCES	28

LIST OF TABLES

	Page
Table 1: Putative Temperature Sensing Genes	16
Table 2: RNAi Knockdown of Temperature Genes	17

LIST OF FIGURES

	Page
Figure 1: Rotifer Survival at Five Temperatures	9
Figure 2: The Effects of Hatching Temperature on Survival	10
Figure 3: The Effects of Low Temperature at Different Life Stages	11
Figure 4: The Effects of Intermittent Low Temperature Exposure	12
Figure 5: The Effects of Different Temperatures during Early Life	13
Figure 6: The Effects of Temperature on Asexual Reproduction	14
Figure 7: The Effects of Temperature on Stress Resistance	15
Figure 8: The Effects of Gene Knockdown on Survival at Different Temperatures	18

LIST OF ABBREVIATIONS

RNAi	RNA Interference
dsRNA	Double-Stranded RNA
PKC1	Protein Kinase C 1
PKC2	Protein Kinase C 2
S6P	Ribosomal Protein S6 Polypeptide 2
S6K α	Ribosomal Protein S6 Kinase Alpha-1
TRP1	Transient Receptor Potential Cation Subfamily Member 1
TRP7	Transient Receptor Potential Cation Subfamily Member 2
FhTF	Forkhead Transcription Factor n1 4 Transcript Variant 1
FhBC	Forkhead Box C-like Protein
FhBJ	Forkhead Box j2 3 Transcription Factor
YBoxF	Y-Box Factor
YBoxBP	Y-Box Binding Protein 1
MERP	Mammalian Ependymin-related Protein

SUMMARY

Environmental temperature greatly affects lifespan in a wide variety of animals, but the exact mechanisms underlying this effect are still largely unknown. A moderate temperature decrease from 22°C to 16°C extends the lifespan of the monogonont rotifer *Brachionus manjavacas* by up to 163%. Thermodynamic effects on metabolism contribute to this increase in longevity, but are not the only cause. When rotifers are exposed to 16°C for four days and then transferred to 22°C, they survive until day 13 at nearly identical rates as rotifers maintained at 16°C continuously. This persistence of the higher survival for nine days after transfer to 22°C suggests that low temperature exposure alters the expression of genes that affect the rate of aging. The relative persistence of the gene regulation effect suggests that it may play an even larger role in slowing aging than the thermodynamic effects. The life extending effects of these short-term low temperature treatments are largest when the exposure happens early in the life cycle, demonstrating the importance of early development. There is no advantage to lowering the temperature below 16°C to 11° or 5°C. Rotifers exposed to 16°C also displayed increased resistance to heat, starvation, oxidative and osmotic stress. Reproductive rates at 16°C were lower than those at 22°C, but because they reproduce longer, there is no significant change in the lifetime fecundity of females. To investigate which genes contribute to these effects, the expression of specific temperature sensing genes was knocked down using RNAi. Of 12 genes tested, RNAi knockdown of four eliminated the survival enhancing effects of the four-day cold treatment: TRP7, forkhead box C, Y-box factor, and ribosomal protein S6. This demonstrates that active gene

regulation is an important factor in temperature mediated life extension, and that these particular genes play an integral role in these pathways. As a thermoresponsive sensor, TRP7 may be responsible for triggering the signaling cascade contributing to temperature mediated life extension. The TRP genes may also provide especially promising candidates for targeted gene manipulations or pharmacological interventions capable of mimicking the effects of low temperature exposure. These results support recent theories of aging that claim rate of aging is determined by an actively regulated genetic program rather than an accumulation of molecular damage.

CHAPTER 1

INTRODUCTION

Many environmental and genetic factors interact to affect aging rate, and a deeper understanding of these mechanisms is integral to discovering new interventions to slow the rate of aging (Malgalhaes et al. 2012). Emerging evidence is shifting the traditional views of aging as an accumulation of molecular damage to an actively regulated signaling network (Gems & Partridge 2013). Even small changes affecting the interconnected pathways can lead to large changes in longevity, especially when applied over the entire lifespan (Conti 2008).

Temperature is a key environmental factor that greatly affects lifespan in all animals studied. There are many cases where modestly lower environmental temperatures greatly increase the lifespan of ectotherms. For example, a 5°C decrease in water temperature extends lifespan of a small, South American fish (genus *Cyanolebias*) by nearly 100% (Rikke & Johnson 2004). In another fish model, lowering environmental temperature from 25°C to 22°C caused not only a significant increase in mean and maximum lifespan, but also delayed the onset of aging markers such as diminished cognitive ability and lipofuscin accumulation (Valenzo et al. 2006). In lakes with thermal gradients, *Daphnia* that prefer the colder waters have lower metabolic rates and live several times the usual lifespan (Pietrzak et al. 2013). Because of the internal mechanisms for maintaining a constant temperature, these effects are harder to study in homeotherms (Carrillo & Flouris 2011). However, transgenic mice with a 0.3°C to 0.5°C reduction in core body temperature had 12 to 20% longer lifespans than controls, regardless of diet (Conti et al. 2006).

Although the exact mechanism of temperature mediated life extension remains largely unknown, it is thought to be related to the same mechanisms by which caloric

restriction (CR) extends lifespan. In homeotherms, CR is associated with a lower core body temperature because it lowers metabolic rates (Conti 2008). The magnitude of the body temperature response to CR varies with different genetic strains of mice (Rikkel & Johnson 2004). In the fish *Cynolebias*, CR combined with reduced environmental temperature produced an additive effect on longevity, extending lifespan by nearly 300% (Rikke & Johnson 2004). Several models suggest overlapping mechanisms by which CR and body temperature affect longevity. These include genetic regulation, reductions in inflammatory response, decreased oxidative damage, increased metabolic efficiency, and hormone regulation (Carrillo and Flouris 2011; Conti 2008).

These regulatory pathways are likely highly interconnected and active gene regulation seems to play a major role in temperature mediated extension of longevity (Lee and Kenyon 2009). It is also appreciated that passive thermodynamic effects contribute to the temperature effect, but how much remains to be quantified. In *C. elegans*, it was found that life extension by lower environmental temperatures is dependent on a thermosensitive genetic pathway, a TRP channel, a calcium sensitive PKC, SGK, and the DAF-16/FOXO transcription factor (Xiao et al. 2013). The DAF-16/FOXO pathway is linked to the nutrient sensing pathway, further connecting the mechanisms of temperature and CR. TRP channels are further known to play a role in external and internal environmental sensing and longevity regulation (Linford et al. 2011). Additionally, the expression of many genes is known to be regulated by changes in temperature. In zebrafish, a shift from 25°C to 12°C increased expression of ependymin, suggesting a role in cold acclimation (Tang et al. 1999). In the rotifer *Brachionus manjavacas*, a one hour 40°C heat shock increased the expression of four heat shock proteins (HSPs) which play an essential role in thermotolerance and stress protection (Smith et al. 2012). In plants, a 15 minute exposure to 4°C increases the expression of CBF (cold-repeat binding factors), transcriptional factors that trigger a signaling cascade inducing freezing tolerance (Thomashow 2010).

Members of the phylum Rotifera are a useful model for studying aging mechanisms (Snell 2014). Rotifers are members of the understudied supra-phylum Lophotrochozoa. They have a short lifespan of about 12 days at 22°C, which allows full life table experiments to be performed in a short time (Snell et al. 2014 a). RNAi can be used to effectively knockdown gene expression of selected genes, and biological effects can be observed to better understand the pathway or mechanism by which these genes are acting (Snell et al 2014 b). In this study, we use the rotifer *B. manjavacas* to explore the effects of different temperature treatments on longevity and identify genes important to temperature-mediated changes in lifespan. We hypothesized that exposure to lower temperatures would greatly extend rotifer lifespan, through both thermodynamic effects and gene regulation. Thermodynamic effects alone cannot account for the observed life extension. We also hypothesized that knockdown in the expression of temperature sensing genes can lead to large changes in longevity and reproduction.

CHAPTER 2

MATERIALS AND METHODS

Rotifer Culture

All experiments were performed using the rotifer species *Brachionus manjavacas*. This strain was originally collected from the Azov Sea in Russia, and has been continuously propagated in the laboratory since 1983, with resting eggs being periodically collected, dried, and stored. Before each experiment, *B. manjavacas* resting eggs were hatched in 25mL, 15ppt artificial sea water (ASW, Instant Ocean) under constant fluorescent illumination (2000 lux) at 25°C. Under these conditions, hatching begins after 18-20 hours, resulting in a uniform cohort of neonates. For particular treatments, resting eggs were hatched at 16°C. At this temperature, hatching was delayed until 48 hours. Hatchlings were fed the green alga *Tetraselmis suecica* cultured in modified F medium (Guillard 1983) in a 560mL chemostat with 25% daily medium replacement, at 25°C and constant fluorescent illumination of 2000 lux.

Experimental Design and Treatments

Full cohort life table experiments were conducted with 120 newly hatched female *B. manjavacas* per treatment. Animals were kept in 24-well plates with each well containing 5 females in 1mL of medium. Medium contained 6×10^5 *T. suecica* cells/mL in 15ppt ASW and 20 μ M 5-fluoro-2-deoxyuridine (FDU), added to prevent the hatching of asexual eggs (Snell et al 2014b). Plates were checked daily and mortality was recorded until all animals were dead. All animals were transferred to new plates with fresh medium every 8 to 16 days as needed to replenish food supply.

Reproductive life table experiments were performed in a similar fashion, with a few modifications. Single females were kept in each well of 24-well plates in 1mL of medium, for a total of 24 females per treatment. Medium contained 2×10^5 *T. suecica* cells/mL in 15ppt ASW, and no FDU was added to allow normal egg hatching. Offspring were produced parthenogenetically and were counted and removed daily. The original maternal females were transferred to new plates every 6 to 12 days, depending on food supply.

All plates were kept in the dark at the appropriate temperature in Percival I-41VL incubators. Standard control temperature was 22°C, with low temperature treatments of 20, 18, 16, 11 and 5°C. The plates were removed from the incubators once a day and scored at room temperature (22°C). Care was taken to minimize the time the plates were removed from the appropriate temperature treatment.

Stressor Challenge Experiments

Five stressor challenge experiments were conducted to test the effects of low temperature exposure on resistance to starvation, oxidative stress, UV exposure, osmotic shock, and heat shock. All animals were kept in 6-well plates with each well containing several dozen animals in 5mL medium. Medium consisted of 6×10^5 *T. suecica* cells/mL in 15ppt ASW with 20μM FDU. Plates were stored in the dark at 16°C for either 0, 1, 2 or 4 days before being transferred to 22°C. On the fourth day, animals were rinsed with 15ppt ASW to clear away algae, and challenged with one of five stress treatments.

Rotifers in the starvation treatment were transferred to 15ppt ASW and deprived of food for 72 hours. Osmotic shocked animals were transferred from 15ppt to 60ppt ASW in a small petri dish for 1 hour, then returned to 15ppt. Heat shocked animals were transferred to a petri dish where they were incubated at 40°C for 1 hour then returned to 22°C. UV stressed animals were transferred into a small petri dish in 5mL 15ppt ASW and exposed to UV-B radiation for 20 minutes. The dish was 25cm from an 8W light

source (UVP, model UVM-28EI) with an intensity peak at 302nm. After undergoing these stress treatments, these animals were transferred to 24-well plates with a total of 120 animals per treatment. Each well consisted of 10 animals and 1mL of 15ppt ASW with 20 μ M FDU. In the oxidative stress treatment, the medium in the plates also contained 0.1 μ M juglone. These stress treatments were chosen to produce considerable, but not 100%, mortality in the control. All plates were stored in the dark at 22°C, and scored for survival after 72 hours. Survival is reported as the average percentage surviving in each treatment.

Synthesizing and Purifying dsRNA

Genes putatively related to temperature sensing pathways were derived from the *B. manjavacas* Transcriptome Shotgun Assembly Project. These sequences are available at DDBJ/EMBL/GenBank under the accession GARS01000000. Twelve genes were selected based on sequence similarity to genes identified by Xiao et al (2013) and other temperature sensing pathways. Primers were designed to amplify a 500bp region from each rotifer gene, and DNA product was sequenced using Eurofins Genomics SimpleSeq to ensure proper amplification of the genes of interest. Primers and accession numbers for all genes can be found in Table 1.

To collect DNA for dsRNA synthesis, 0.5 μ M of forward and reverse primers with a T7-motif (TAATACGACTCACTATAGG) were used with Go-Taq DNA polymerase (Promega), 1mM MgCl₂, 100mM dNTP (Promega), 10x buffer, and 40ng of genomic *B. manjavacas* DNA. The resulting PCR products were purified using a QIAquick PCR purification kit (Qiagen). dsRNA was synthesized from the T7 tagged DNA and purified using a MEGAscript® RNAi kit (Invitrogen). Purified dsRNA was quantified using a NanoDrop 1000 (Thermo Scientific) and visualized on a 1% agarose gel to verify band size.

To serve as a control for mortality or changes in expression due to exposure to transfection reagents, a scrambled sequence of the rotifer TOR gene was synthesized by the company IDT and used in place of genomic DNA in the initial PCR, as described by Snell et al. (2014). The scramble sequence is not found in the known *B. manjavacas* genome, and was used as a control in all RNAi experiments.

RNAi Knockdown Experiments

Four types of RNAi experiments were performed to screen for biological effects of gene expression knockdown. The biological effects tested were ability of resting eggs to hatch, female survival, reproduction, and cold sensitivity. For all experiments, a transfection solution was prepared with 47uL PBS, 1uL FuGene6 (Promega), and 2uL (200ng) dsRNA and incubated at room temperature for 20 minutes (modified from Snell et al. 2010). For hatching, survival, and cold sensitivity tests, *B. manjavacas* neonates hatching from resting eggs were collected within 2 hours of their births, and transferred to 500uL 15ppt ASW in a 24-well plate. Roughly 100 animals were transferred to each well. 50uL of transfection solution was added to each well, and the plate was incubated at room temperature for 4 hours before animals were transferred to each experimental treatment.

For the survival experiments, animals were transferred to 24-well plates with 1mL 6×10^5 *T. suecica* cells/mL, 20uM FDU, and 5 females in each well. One full plate was used for each treatment, and plates were incubated at 28°C in the dark for eight days. On days 2, 4, and 6, plates were treated with 5uL additional FDU (1mg/mL) to prevent asexual egg hatching, and on day 6 plates were fed 100uL of 6×10^6 *T. suecica* cells/mL per well. After 8 days, the number of living animals was counted and survival was recorded as average percent surviving in each treatment.

In the reproductive experiments, animals were transferred from the transfection solution into 24-well plates with 1mL 2×10^5 *T. suecica* cells/mL and 1 female in each

well. One full plate was used for each treatment, and the plates were incubated in the dark at 25°C for 72 hours. Offspring were counted and removed daily, and population growth rate r was calculated at 72 hours.

To screen for changes in lifespan due to cold sensitivity, animals were transferred into 24-well plates with 1mL 6×10^5 *T. suecica* cells/mL, 20μM FDU, and 5 females in each well. For each treatment, 3 full plates were prepared. One of these plates was incubated at 22°C continuously, another was incubated at 16°C continuously, and the third was incubated at 16°C for the first four days and then transferred to 22°C. On days 4, 8, and 12, each well was treated with 5uL additional FDU (1mg/mL), and on day 8 each well was fed 100uL 6×10^6 *T. suecica* cells/mL. Survival was recorded daily until day 15, and the data was used to generate right-censored survival curves.

To test the effects of RNAi knockdown on hatching, diapausing *B. manjavacas* embryos (resting eggs) were decapsulated using the protocol of Snell et al. (2010), and then transferred to 24-well plates with 500uL 15ppt ASW, 25uL transfection solution, and 20 resting eggs in each well. Six replicate wells were used for each treatment. Plates were incubated at 25°C under constant fluorescent illumination (2000 lux). Number of eggs hatched in each well was recorded after 48 hours.

Statistics

Survival curves from full life tables and RNAi cold sensitivity screens were evaluated using the JMP Pro 11 (SAS Institute) reliability and survival analysis with a Wilcoxon's test to compare control and treatments. Stress challenge, hatching, 8 day survival, and reproductive screens were compared by ANOVA with Dunnett's test to compare treatments to control.

CHAPTER 3

RESULTS

Small changes in environmental temperature lead to large changes in rotifer lifespan (Figure 1). A 2°C decrease from 22°C to 20°C extended lifespan by 39%. A further 2°C decrease to 18°C extended lifespan by 83%, and a 6°C decrease from 22°C to 16°C extended lifespan by 115% compared to the 22°C control. Similarly, a 6°C increase in temperature to 28°C decreased lifespan by 52%. All of these temperature induced changes in survival are significantly different from the 22°C control ($P < 0.0001$) by a Wilcoxon test.

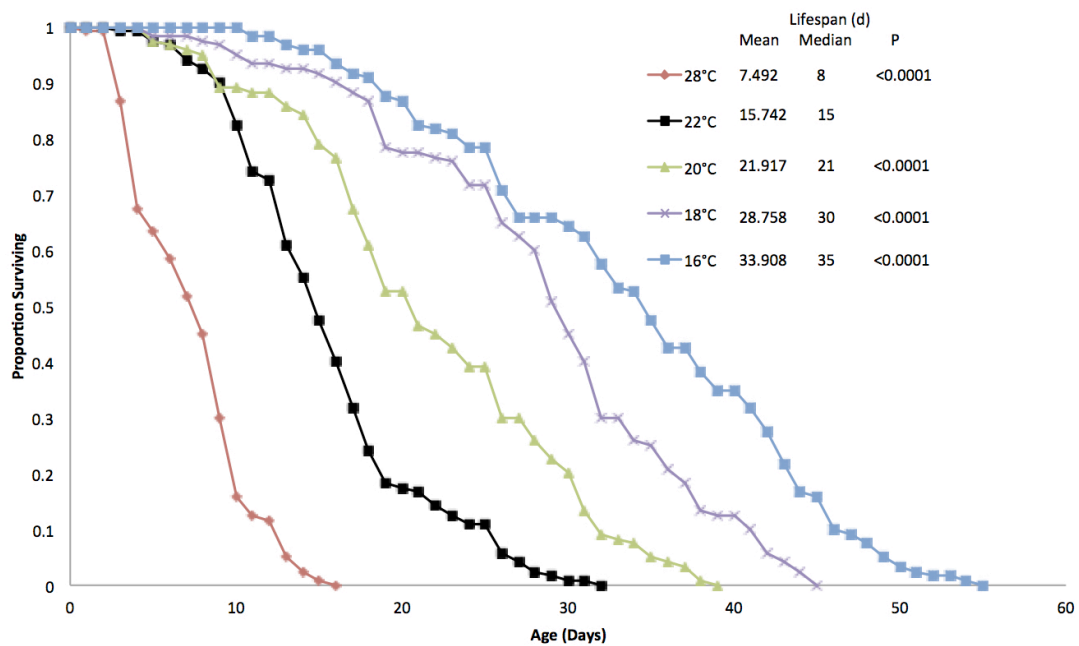


Figure 1. Survival curves for *B. manjavacas* incubated at 5 different temperatures. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22°C control.

A second experiment examined the effects of hatching the resting eggs at different temperatures on adult survival and lifespan (Figure 2). Hatching resting eggs at 16°C and incubating the newborns at 22°C produced lifespans not significantly different from control animals hatched at 25°C and incubated at 22°C. Animals hatched at 25°C and incubated at 16°C produced significant life extension of 63% as compared to control. However, animals that were hatched at 16°C and maintained at 16°C experienced an 86% longer lifespan, significantly greater than rotifers hatched at 25°C and transferred to 16°C.

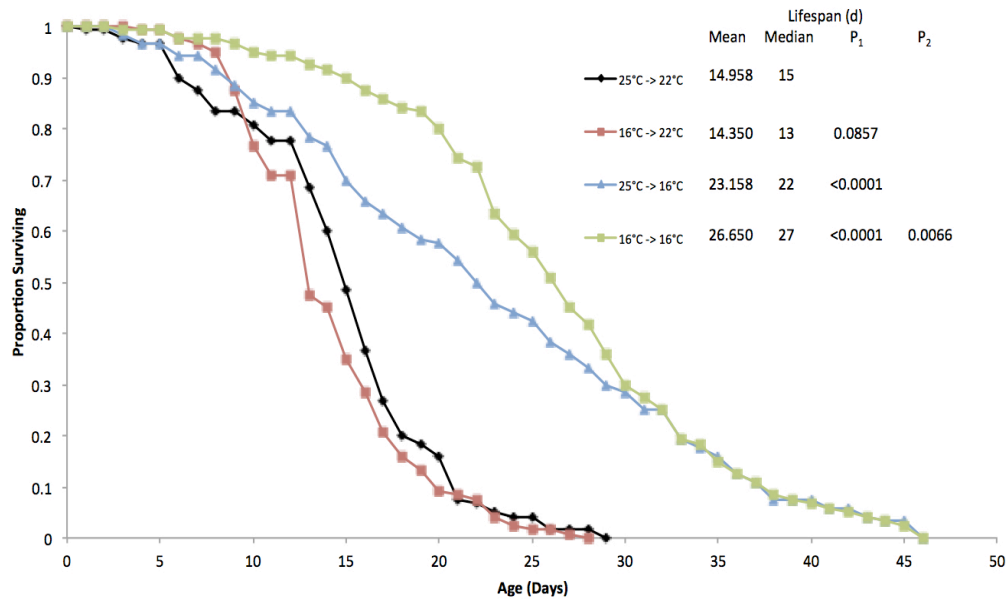


Figure 2. Survival curves for *B. manjavacas* hatched and incubated at different temperatures. The first number of the treatment refers to the temperature resting eggs were hatched in and the second refers to the incubation temperature after hatching. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days. P₁ represents the Wilcoxon test probability that the treatment curves are similar to the 25°C->22°C control, and P₂ represents the Wilcoxon test probability that the curve is similar to the 25°C->16°C treatment.

A third life table experiment was performed to explore the effects of 16°C exposure at various life stages (Figure 3). Continuous 16°C exposure extended lifespan by 163%. Exposing rotifers to 16°C for only their first four days significantly extended lifespan by 31%. Even though exposure to 16°C only lasted until day four, the survival curve closely followed that of the 16°C continuous treatment until day 13. Exposing the animals to 16°C from days 4 to 8 also extended lifespan by 16%, but was not nearly as effective at extending lifespan as the same exposure in the early age classes. Exposure to 16°C from days 8 to 12 provided no significant life extension.

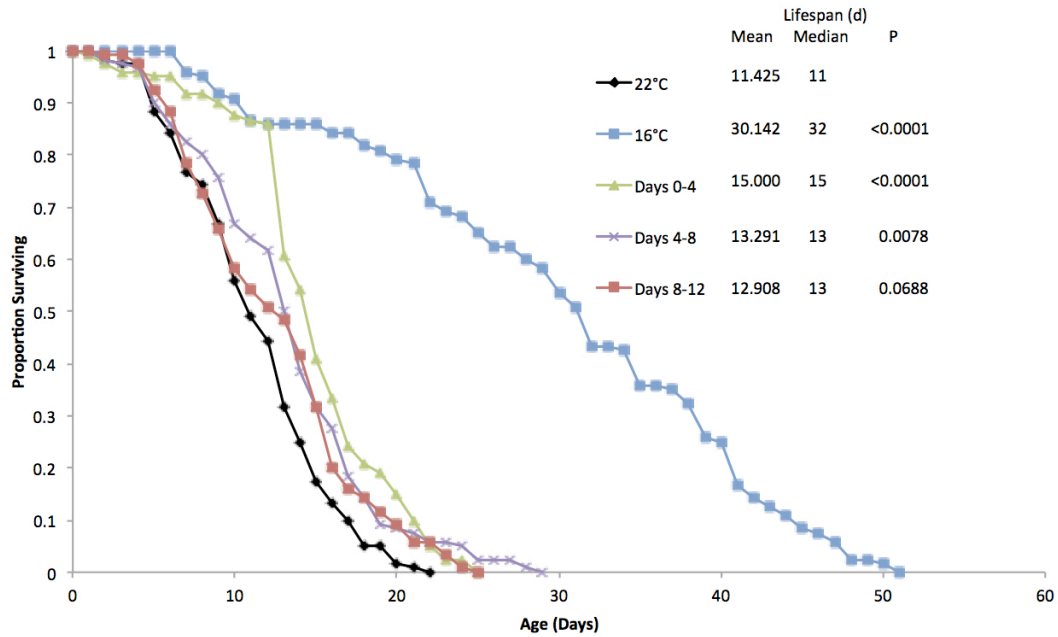


Figure 3. Survival curves for *B. manjavacas* incubated at different temperatures during different stages of the lifespan. The first two treatments remained at a single continuous temperature. The other three treatments were incubated at 16°C for the days labeled and 22°C for all other days. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22°C continuous control.

A fourth life table experiment was performed to investigate whether additional exposure to 16°C could extend the protective effect of the initial four day 16°C exposure (Figure 4). In this experiment, 16°C exposure for the first four days did not significantly increase median lifespan, but survival was essentially the same as the 16°C continuous treatment until day 11. However, a 16°C exposure for the first four days, followed by alternating four days at 22°C and then four days at 16°C for the remainder of the lifespan significantly extended lifespan by 24%, and the survival curve followed that of the 16°C continuous treatment until day 16.

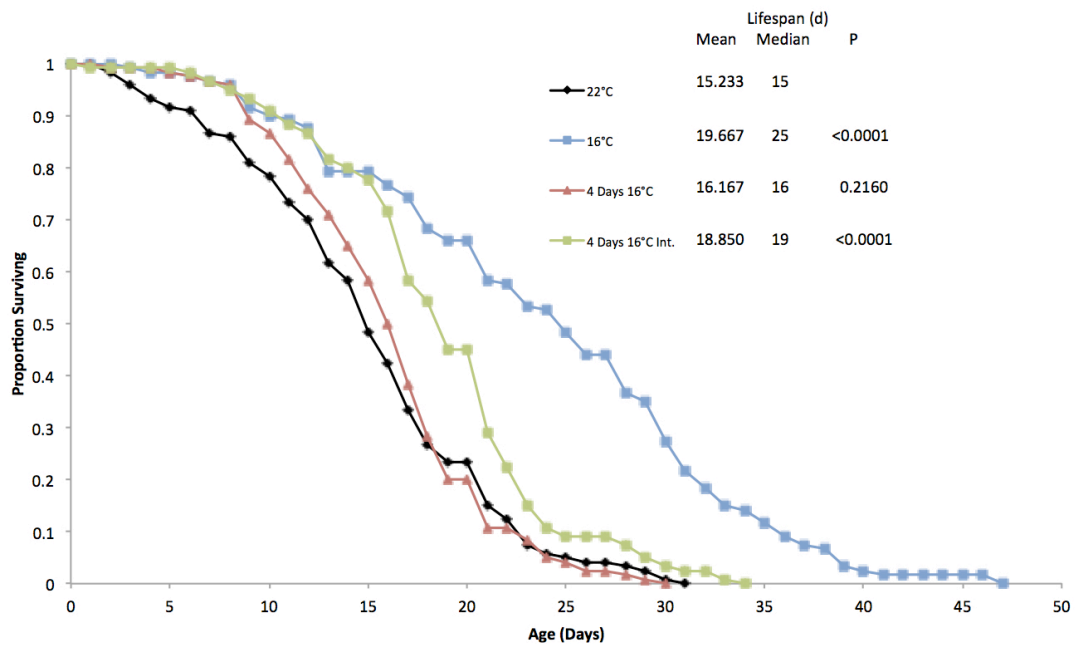


Figure 4. Survival curves for *B. manjavacas* incubated at different treatments . 4 Days 16°C was exposed to 16°C only for the first 4 days of life, while the Int. treatment was exposed for 4 days intermittently throughout the lifespan. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22°C control.

The effects of a range of early life cold treatments were examined in another life table (Figure 5). In this experiment, a four day exposure to 16°C extended lifespan by 22%. A four day exposure to 11°C similarly extended lifespan by 26%, but provided no significant additional benefit beyond exposure to 16°C. A four day exposure to 5°C on the other hand, provided no life extension, and the survival curve displayed an unusually large number of deaths in the early age classes. The rotifers exposed to 5°C were also on average of 42% smaller than those incubated at 22°C, and in many of them, the pseudocoelom was dark green, suggesting that the gut ruptured allowing algae to escape into the pseudocoelom.

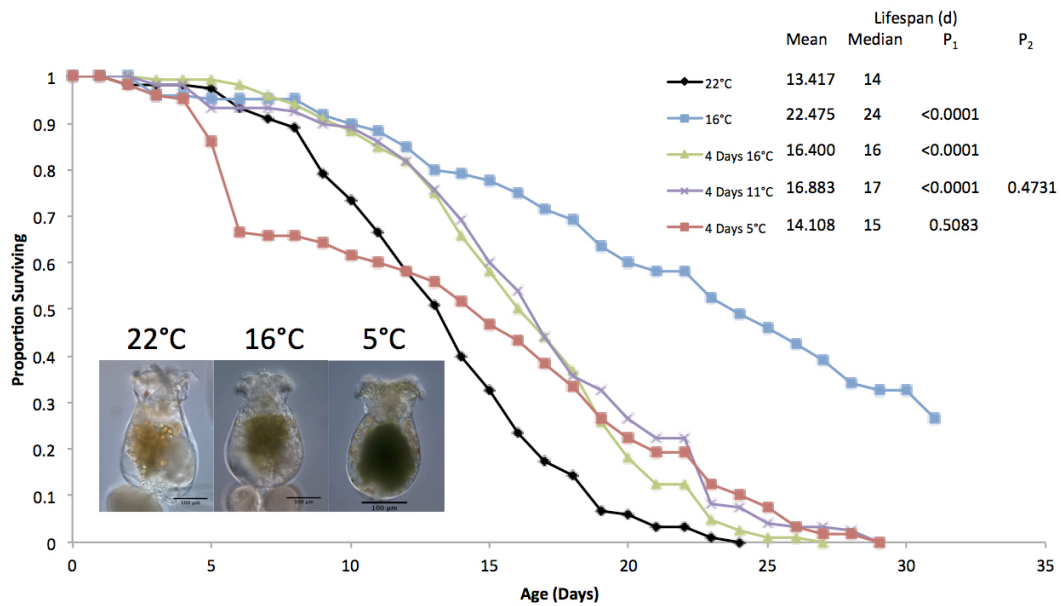


Figure 5. Survival curves for *B. manjavacas* incubated at different temperatures for the first four days of the lifespan. All four day treatments were transferred to 22°C on day 5. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age. Mean and median lifespan are reported in days. P₁ represents the Wilcoxon test probability that the treatment curves are similar to the 22°C control, and P₂ represents the Wilcoxon test probability that the curve is different from the 4 days at 16°C treatment. Photos show 5 day old animals from different temperatures.

A reproductive life table was performed to observe the effect of a 16°C temperature on lifespan and reproduction. In the absence of FDU, the 16°C treatment extended lifespan by 122% as compared to the 22°C control (Figure 6a). The reproductive curves of the two treatments were very different, with the 22°C treatment peaking at nearly 7 offspring per female per day and earlier (day 4), whereas the 16°C treatment produced fewer offspring (peak: 2.5 offspring per female per day, days 6-8) for a longer period of time (Figure 6b). Nonetheless, the mean total offspring produced by a female over her lifespan (R_0) was not significantly different between the two temperatures. The average proportions of the life span spent in pre-reproductive, reproductive, and post-reproductive stages also did not differ significantly between treatments.

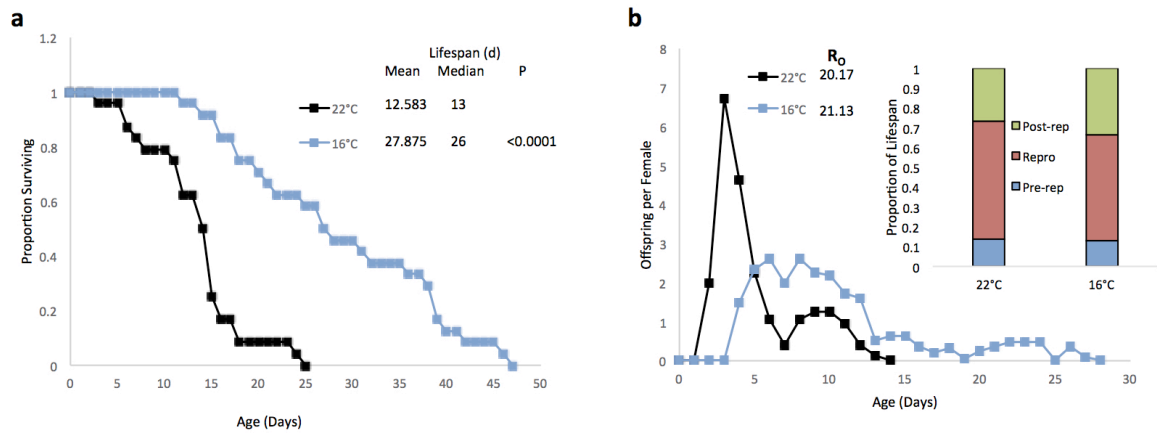


Figure 6. a) Survival curves for *B. manjavacas* incubated at 2 different temperatures. b) Reproductive curves for *B. manjavacas* incubated at 2 different temperatures. Proportion surviving represents the fraction of an initial cohort of 24 females surviving to each age. Mean and median lifespan are reported in days, and P represents the Wilcoxon test probability that the treatment curves are similar to the 22°C control. Offspring per female refers to the average number of offspring produced each day by a cohort of 24 females. R_0 is the mean total offspring produced by a female over the lifetime. Pre-rep refers to the average proportion of the lifespan a female spends in the pre-reproductive stage, Repro is the proportion spend reproducing, and Post-rep is the proportion spent in the post-reproductive stage.

Another effect of exposure to 16°C was to provide protection from different stressors, as demonstrated in five stress challenge experiments (Figure 7). Four days of continuous 16°C exposure provided significant protection from all five stressors as compared to animals incubated at 22°C. One or two days of 16°C exposure followed by 22°C incubation until the stress tests on day 4 also provided significant protection from oxidative, osmotic, and heat stress. Two days of 16°C exposure provided protection from starvation, but only one day exposure did not. Neither one nor two days of 16°C treatment was enough to provide protection from UV light.

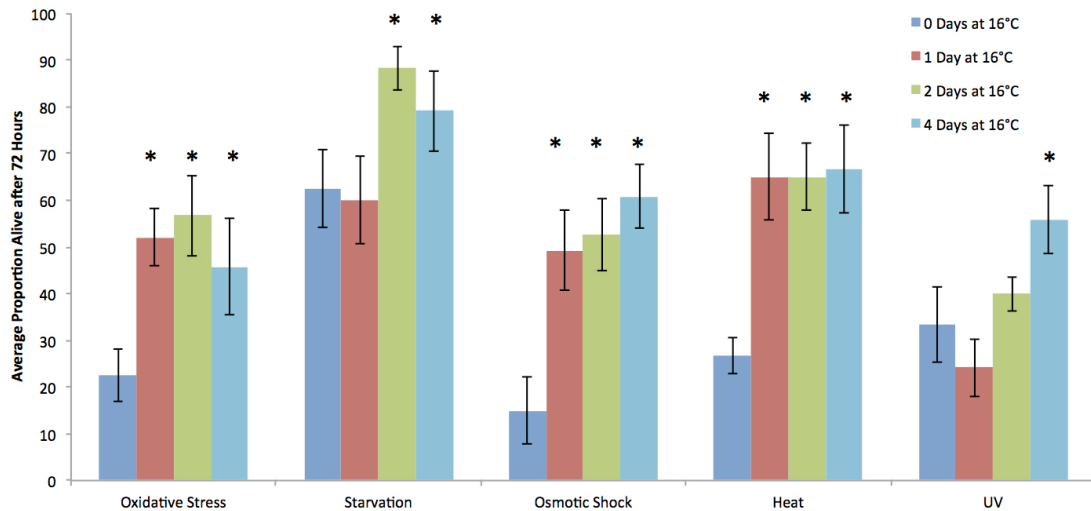


Figure 7. The effects of different stressors on *B. manjavacas* survival. All treatments were incubated at 22°C when not in the 16°C treatment specified, and all stress challenges were applied on day 4. Oxidative stress exposed animals to 0.1μM juglone for 72 hours. Animals in the starvation test were removed from all food for 72 hours. Osmotic shock exposed animals to 60ppt ASW for 1 hour. Heat shock exposed animals to 40°C for 1 hour. UV treatment animals were exposed to UV light for 20 minutes. Variance is represented by the standard error bars on each column. Asterisks denote a P-value of <0.05 calculated from an ANOVA with Dunnett's test comparing the treatments to the control for each stressor.

RNAi was used to explore the importance of potential temperature sensitive genes in mediating these life extension effects. Twelve rotifer genes were chosen based on sequence similarity to genes known to be involved in temperature sensing pathways in *C. elegans* (Table 1). A series of screens were used to assess the different types of biological responses to RNAi knockdown of these genes (Table 2). Only PKC1 knockdown had any significant effect on resting egg hatching, decreasing the mean number of eggs hatched after 48 hours by 73%. RNAi knockdown of PKC1, S6P, TRP7 and YBoxBP genes all significantly decreased average number of offspring produced by asexual mothers after 72 hours. Most interestingly, S6K α , TRP7, and FhBC knockdown increased mean survival after eight days at 28°C by 36%, 23% and 29%, respectively.

Table 1. List of putative temperature sensing genes and the primer sequences used in PCR. T7 primers contain an additional T7 adapter at the 5' end. All genes can be found within the *Brachionus manjavacas* transcriptome shotgun assembly (GARS01000000) using the accession numbers listed.

Gene	Abbreviation	GB Accession	Related Gene/Pathway	Forward	Reverse
Protein Kinase C 1	PKC1	GARS01002868.1	SGK (Human)	GGCTGGTTCAAAGTGTGGT	ATCGCCACCGTTGATGATT
Protein Kinase C 2	PKC2	GARS01002161.1	PKC (Human)	TTGAAGAATTGCGTCACAGG	CGCACATTGTAGTCTTGGA
Ribosomal Protein S6 Polypeptide 2	S6P	GARS01003002.1	SGK (Human)	GCGATGACGACAATATCAG	TCCAATGCATGAACAGCTC
Ribosomal Protein S6 Kinase Alpha-1	S6Ka	GARS01002535.1	SGK (Human)	CAAGCCGACTCCATCTCATT	CAGGCTTTAGATCGCGGTAA
Transient Receptor Potential Cation Subfamily Member 1	TRP1	GARS01002238.1	TRP (Human)	GCTGGCCAGACAAATACTCG	ATACGCCGAAAGCAAAGAAA
Transient Receptor Potential Cation Subfamily Member 7	TRP7	GARS01012197.1	TRP (C elegans)	CAATGTTTTGGTCCTTATTTGG	CCAAATGGAATTGATCCGATA
Forkhead Transcription Factor n1 4 Transcript Variant 1	FhTF	GARS01002302.1	Daf 16-g (C elegans)	CCCAAGTGGCTATGAGAAGC	TTAGCTTGCCTGACAGGTG
Forkhead Box C-like Protein	FhBC	GARS01006072.1	Daf 16-a (C elegans)	AACGACTGCTTCTGCAAGGT	GACCGAAAACGACGAGTAGG
Fork-head Box j2 3 Transcription Factor	FhBJ	GARS01005585.1	Daf 16-h (C elegans)	CATCAATGGAATGGTTGTCG	AACCTTTCCAGGGTCATCT
Y-box Factor	YBoxF	GARS01001540.1	Temperature Sensitivity	TGTCGGATACCGCAGTACAA	TTTGGTGTGTCACGGTTTA
Y box Binding Protein 1	YBoxBP	GARS01002878.1	Temperature Sensitivity	TTGAACCAAGTGCCCAACAA	GGCTGTCTTGGTGTCTGTT
Mammalian Ependymin-related Protein	MERP	GARS01009784.1	MERP (D rerio)	ACCATGCACATCTCCTCTC	CGATTCTGGAACAATGTCTG

Table 2. The effects of RNAi mediated knockdown of temperature related genes on *B. manjavacas*. Hatching refers to the average number of resting eggs hatched after 48 hours. Reproduction refers to average number of offspring per female at 72 hours. Survival refers to the average proportion of animals surviving after 8 days at 28°C. NS denotes no significance difference from the scramble treated control ($P>0.05$) as calculated by ANOVA with Dunnett's test. Percentages refer to significant increase or decrease as compared to control.

Gene	Hatching	Reproduction	Survival
PKC1	↓ 72.5%	↓ 15.8%	NS
PKC2	NS	NS	NS
S6P	NS	↓ 16.1%	NS
S6Ka	NS	NS	↑ 36.2%
TRP1	NS	NS	NS
TRP7	NS	↓ 17.2%	↑ 23.2%
FhTF	NS	NS	NS
FhBC	NS	NS	↑ 29.0%
FhBJ	NS	NS	NS
YBoxF	NS	NS	NS
YBoxBP	NS	↓ 21.9%	NS
MERP	NS	NS	NS

To assess the role of these genes in rotifer temperature sensing more specifically, screens were conducted to compare the survival of RNAi treated animals at 22°C continuous, 16°C continuous, and a four day 16°C exposure before being transferred to 22°C (Figure 8). Control animals were treated with a scrambled dsRNA from the rotifer TOR gene. A Wilcoxon's test showed that both the four day and continuous 16°C treatments have very similar survival, and that both treatments survived significantly better than the 22°C treatment. However, four of the RNAi knockdown treatments markedly altered the survival curves. When rotifers were exposed to dsRNA of TRP7, FhBC, YBoxF, and S6P genes, the four day 16°C exposure curve showed significantly lower survival than the 16°C continuous, and was much more similar to 22°C continuous exposure. This shift in the effect of a 16°C exposure on rotifer survival demonstrates that these four genes likely have a key role in sensing and responding to low temperatures by rotifers.

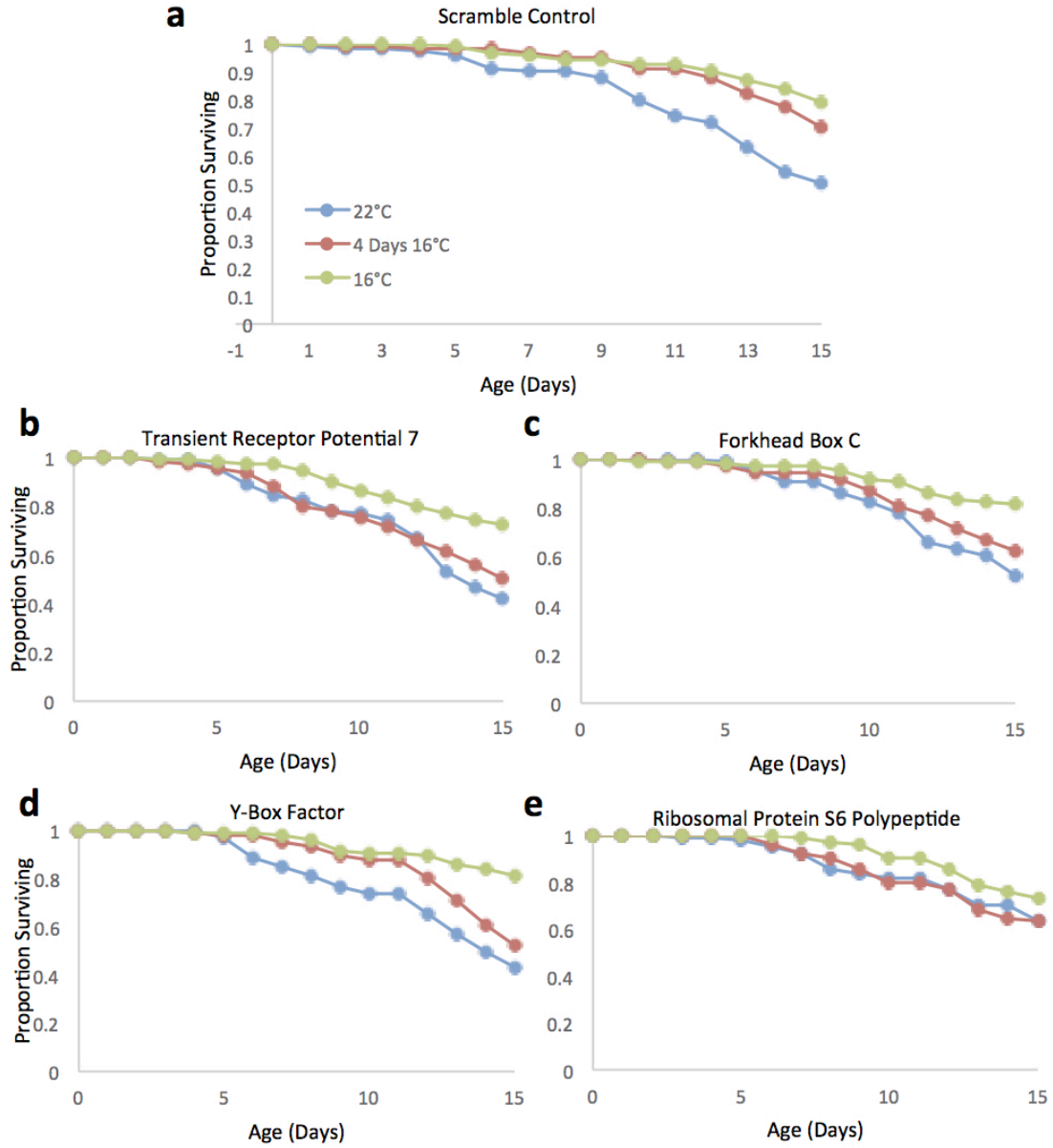


Figure 8. Survival curves for RNAi treated *B. manjavacas* incubated at 3 different temperatures. a) Typical curves for the scramble treated control animals at each temperature. b-e) Survival curves for expression knockdown treatments where the persistent survival effect of a 4 day 16°C exposure is eliminated. Proportion surviving represents the fraction of an initial cohort of 120 females surviving to each age.

CHAPTER 4

DISCUSSION

The initial life table experiment demonstrated that even modest changes in temperature (9-27%) could greatly extend rotifer lifespan (Figure 1). Each 2°C reduction in temperature resulted in roughly 40% lifespan extension. A 6°C temperature difference from 22°C to 16°C provided the greatest lifespan increase up to 163%. Although a 6°C difference in core body temperature would be sizeable in a homeotherm, it lies well within the thermal tolerance range for a *Brachionus* rotifer (Walker 1981).

It is well documented that across a variety of species including microbes, ectotherms, and endotherms, metabolic rate decreases with decreasing temperature by reducing the rates of biochemical reactions (Gillooly et al 2001). In temperate plants, lowered environmental temperature also signals changes in hormone levels and molecular chaperones that assist with both post-transcriptional and post-translational modifications (Miura and Furumoto 2013). These changes contribute to cold acclimation and enhance freezing tolerance. In tropical plants, temperatures below 15°C disrupt vital physiological processes including photosynthesis and respiration, slowing and in some cases completely inactivating metabolism (Lutkatkin et al 2012). Insects are able to rapidly acclimate to cold temperatures through a variety of physiological mechanisms (Teets and Denlinger 2013). These responses include the accumulation of cryoprotectants such as glycerol, a metabolic shift towards glycolysis, increases in membrane phospholipid unsaturation, and changes in calcium levels (Lee and Denlinger 1991; Teets and Denlinger 2013; Murray et al 2007). However, these responses alone are not likely sufficient to explain the life extension effects found in our experiments.

A key finding from this study is the persistence of higher survival after removal from the 16°C treatment. Survival rates for the four days at 16°C treatment matched that of continuous 16°C exposure until day 12, even though the former treatment was exposed to 22°C days 5-12. This demonstrates that the mechanism of low temperature life extension cannot solely be a passive thermodynamic effect. It is quite likely that low temperature exposure modulated the expression of specific genes, thereby slowing aging processes. Clearly, identifying these genes and how they impact aging processes is a high priority in future work. Based on the duration of this persistent survival effect, and our findings that knockdown of specific genes can completely eliminate this effect, it is possible that the genetic regulation elicited by changes in temperature play an even larger role in temperature mediated life extension than passive thermodynamic effects. However, quantifying the relative magnitude of each mechanism requires additional research.

Although complete enumeration of the genetic pathways responsible requires further investigation, the life extending responses to lowered temperature are likely caused by a complex signaling cascade regulated by a sensory receptor. The mechanism of temperature sensation in plants remains incompletely understood, but within only 15 minutes of a 4°C exposure, CBF transcription factors are induced. These transcription factors signal a large cascade of other genes involved in cold acclimation and stress resistance (Thomashow 2010; Miura and Furumoto 2013). In *C. elegans*, temperature mediated life extension was found to be dependent on the expression of the TRP channel TRPA-1 (Xiao et al 2013). The TRP superfamily is comprised of a large number of cation channels with a variety of selective activation mechanisms. Many of the TRPs are thermosensory receptors that respond to a wide range of temperatures to trigger signaling cascades that regulate body temperature and avoid tissue damage (Flockerzi and Nilius 2007). TRP thermoreceptors likely control this temperature mediated signaling cascade in

rotifers as well. Because of the linkage between temperature, aging, and metabolism, it is likely that part of this cascade involves nutrient sensing pathways like TOR as well.

A series of RNAi experiments was used to further explore which genes may play an integral role in the thermosensory life extension of rotifers. The genes investigated in these experiments were all derived from the *B. manjavacas* transcriptome based on sequence similarity to genes found in temperature sensing pathways from other animals such as *Caenorhabditis*, *Danio*, and humans. The high similarity between these genes suggests that they have been highly conserved evolutionarily and likely play an important role in metabolism. Of the 12 genes knocked down, only three (TRP7, FhBC, and S6Ka) caused a significant change in survival, and these effects were not nearly as large as the effects produced by a continuous 16°C exposure (Table 2). Changing the expression of only one gene at a time was not sufficient to mimic the effects of a continuous low temperature treatment, further supporting the idea that multiple pathways are involved in temperature mediated life extension.

The persistent effect of a four day 16°C treatment on survival was effectively eliminated in four of the gene knockdown treatments; TRP7, FhBC, YBoxF, and S6P. This emphasizes the likely role of these genes in temperature mediated life extension. FhBC and S6P are related to the DAF-16 and SGK genes, respectively. DAF-16 is a key regulator of both lifespan and stress resistance, and SGK is an upstream regulator of DAF-16 (Riedel et al 2013; Xiao et al 2013). TRP7 is a member of the TRP channel family, which are ancient, multifunctional thermosensory channels that can respond to both extracellular or intercellular activators (Linford et al 2011). TRP7 specifically is related to the *C. elegans* TRPA-1 which detects environmental temperature drops to regulate a signaling cascade (Lee & Kinyon 2009; Xiao et al 2013). Finding ways to artificially manipulate sensory signaling through pharmacological or gene treatments could be a key to capturing the benefits of low temperature treatments on longevity without the need for prolonged low temperature exposure (Linford et al 2011). The TRP

channels provide excellent pharmacological targets for further research because there are several drugs that are already known to bind to mammalian thermosensory TRPs (Flockerzi and Nilius 2007). Further experimentation is necessary to understand these types of gene-environment interactions and to discover new aging interventions (Magalhães et al 2012).

The stress challenge experiments demonstrated that a 16°C temperature treatment provides protection to a variety of stressor types (Figure 7). This further supports the idea that temperature mediated longevity is controlled by a variety of interconnected pathways. Many life-extending interventions are associated with increased stress resistance, so it is not surprising that continuous low temperature exposure induces specific gene expression and provides protection (Johnson et al 1996). However, even a one-day exposure to 16°C provides significant protection against oxidative, osmotic, and heat stress four days later. This provides additional evidence that temperature effects may be caused by the induction of specific genes. There is already considerable support for the idea that stress resistance has a genetic basis and that resistance can arise as an adaptation to abiotic environmental factors (Dragosits et al 2013). In addition, the DAF-16 pathway that has been linked to both temperature mediated lifespan effects and nutrient sensing has also been linked to UV, heat, and hypoxia resistance, further suggesting that temperature responses are mediated by multiple, highly interconnected pathways (Murakami & Johnson 1996; Leiser et al 2011).

Lower temperatures may also confer resistance to stressors through the production of molecular chaperones. Heat shock proteins (HSPs) are well characterized, highly conserved molecular chaperones that are up-regulated after exposure to high temperatures (Fabbri et al 2008). They stabilize proteins and increase thermotolerance in many animals (Smith et al 2012). There is evidence that under cold stress, plants exhibit both post-transcriptional and post-translational regulation. RNA binding proteins prevent RNA from becoming over-stabilized in misfolded conformations at low temperatures, and cold

stress affects an ubiquitin E3 ligase pathway to regulate the proteasome (Miura and Furumoto 2013). In addition, cold stress has been shown to up-regulate HSP70 in *Drosophila* (Sinclair et al 2007). Furthermore, the activation of thermosensitive TRPs in mammalian skin cells has been found to induce the expression of HSPs (Hsu and Yoshioka 2015).

In the reproductive assay, it was found that while an incubation temperature of 16°C increased lifespan by 122% as compared to a 22°C control group, the lifetime fecundity of an individual female was unchanged (Figure 6). Females at 16°C produced fewer offspring per day, but remained reproductive longer. This is consistent with previous research that found that in *B. plicatilis*, increasing temperature increased intrinsic rate of increase, but the effect of temperature on fecundity was negligible as long as the temperature was within the normal range for natural habitats (Miracle & Serra 1989). However, it was also discovered that the slope of the relationship between temperature and reproduction was dependent on the genotype of the strain, and there were distinct differences between warm- and cold-adapted genotypes (Miracle & Serra 1989).

This same phenomenon has been observed between rotifer sibling species in both natural populations and laboratory settings. In Spain, it was found that populations of *B. plicatilis* and two of *B. rotundiformis* were adapted to different temperature and salinity preferences. As such, the three genotypes demonstrated different responses in sexual reproduction to different temperature treatments (Gómez et al 1997; Serra et al 1998). We have observed that the lifespans of three closely related rotifer species (*B. manjavacas*, *B. plicatilis*, and *B. calyciflorus*) each respond differently to 16°C. While the lifespan of *B. manjavacas* increases up to 163% as compared to animals at 22°C, *B. plicatilis* lifespan increased by 84% and *B. calyciflorus* lifespan was increased by only 32% (unpublished observations). These differences in ecological specialization and temperature responses

between closely related species deliver insights into how temperature effects on life history traits can drive rotifer evolution and speciation.

The costs and benefits of cold adaptation have been found to be dependent on environmental conditions. Field tests of *Drosophila* found that cold acclimated flies had better rates of resource acquisition and survival at low temperatures, but were at a large disadvantage at high temperatures (Kristensen et al 2008). It was also found that in *Drosophila*, the benefits of acclimation increased as the severity of the stressor increased (Rako and Hoffmann 2006). However, similar experiments in the Tsetse fly demonstrated no benefits of cold acclimation (Terblanche et al 2007). This is likely due to the fact that the Tsetse fly is a tropical species, and the temperature acclimation provides the most benefit to temperate species that experience seasonal temperature variability (Kristensen et al 2008; Terblanche et al 2007).

Further exploration into the effects of a four day cold treatment on enhanced survival indicated that there was no benefit of reducing temperatures lower than 16°C (Figure 5). A four day exposure to 11°C produced survival nearly identical to a four day 16°C treatment. A four day exposure to 5°C resulted in much poorer survival in early age classes. The rotifers exposed to 5°C were also much smaller and many had algae present in their pseudocoeloms. This suggests disruption of the membrane integrity of the gut, allowing algae to leak into the pseudocoelom. This is consistent with the finding that heat and cold stress caused a decrease in membrane stability in mussels (Yao & Somero 2012).

A life table experiment exploring the effects of intermittent cold treatment demonstrated that enhanced survival from a four day 16°C treatment can be further extended by subsequent intermittent exposure to 16°C (Figure 4). Lifespan was extended in treatments where every four days, the rotifers were transferred from 16°C to 22°C, or vice versa. However, there was no effect of the same transfers being performed every

other day or every two days. This suggests that the regulation of the genes responsible for temperature mediated longevity effects require a specific amount of time to be activated.

Temperature during hatching of *B. manjavacas* is important because this is when embryological development occurs and the rate of aging likely is determined in early life stages (Carrillo & Flouris 2011). Rotifer resting eggs are arrested in the gastrulation stage, so the majority of development occurs during hatching (Boschetti et al 2011). Upon hatching, rotifers are mostly eutelic and there is no further cell replication until new eggs are produced (Wallace 2002). Exposure to 16°C only during these early developmental stages was not enough to significantly extend lifespan, but 16°C exposure during hatching as well as during the full life span increased longevity more than any other treatment (Figure 2). The importance of early development is further highlighted by the fact that a four day exposure to 16°C is enough to extend lifespan only if it occurs in first four days of life (Figure 3). Exposure to 16°C after 8 days of age provided no life extension. These results support the idea that the rate of aging is probably determined early in life.

We also investigated the possible accumulation of glycerol or LEA (late embryogenesis abundant protein) as another mechanism involved in temperature mediated longevity. Treatment with glycerol significantly increases lifespan in *B. manjavacas*, and is commonly used as a cryoprotectant in many different applications (Snell & Johnston 2014). An increase in glycerol levels is also associated with cold acclimation in insects (Lee and Denlinger 1991). LEA is a thermostable protein that protects *B. manjavacas* resting eggs from thermal stress (Jones et al 2012). We tested for changes in endogenous levels of glycerol and LEA at 16°C using the protocols found in Snell & Johnston (2014) and Jones et al (2012), respectively. There was no evidence of the accumulation of either compound.

There are several avenues of investigation into the mechanisms and effects of temperature dependent longevity that would benefit from further exploration. Heat-shock

proteins (HSPs) are another family of genes that should be a high priority for study. HSPs are upregulated in *B. manjavacas* at high temperatures (40°C), and in caloric restriction (Smith et al 2012; Yang et al 2014). Another area for further research is intraspecific differences in temperature response. Different genetic isolates of *B. plicatilis* respond differently to intermittent fasting (Gribble et al 2014). Understanding the basis for the differential response of these naturally occurring strains can provide additional insight into the genetic basis of temperature sensitivity. Also, the RNAi approach taken in this study focused on targeting specific conserved genes. A broader RNAseq approach would provide a more comprehensive look at the genes that are differentially expressed at different temperatures, and could help identify novel genes involved in temperature sensitive pathways.

Analyzing the mechanisms driving temperature mediated life extension is critical to understanding the long-term effects of changes in environmental temperatures. Therapeutic hypothermia in humans where core body temperature is reduced from 37°C to 32-35°C is already being used in clinical applications to treat cardiac arrest and neurological injuries (Oddo et al 2006; Poldrman 2008). Induced hypothermia and hypometabolic states have also long been suggested for use in human space exploration (Cockett & Beehler 1962; Malatesta et al 2007). More research is needed into the effects of periodic cold treatments on aging and lifespan. In addition, global climate change is predicted to have large effects on ectotherms in both aquatic and terrestrial communities (Seifert et al 2015). Research is already being done into temperature driven effects on community dynamics such as predation, extinction, and invasion (Seifert et al 2015). However, it is important to also understand how changes in temperature will affect life history traits such as lifespan and fecundity, which could, in turn, have major impact on community interactions.

Recent theories challenge the traditional view of aging as an accumulation of molecular damage, and instead argue that aging is a result of actively regulated genetic

pathways (Gems and Partridge 2013; Blagosklonny 2012). Our results further emphasize the importance of such genetic regulation. By activating the low temperature-sensing pathway in *B. manjavacas*, we observed enhanced survival even after thermodynamic reductions in metabolism were withdrawn. Exposure to 16°C only enhanced longevity when exposures were during early age classes, supporting theories that the rate of aging is likely determined by environmental factors during early development (Carillo and Flouris 2011). Furthermore, our findings support the idea that many species harbor a latent potential for increased longevity, and that potential can be realized by environmental manipulations (Johnson et al 2006). The many interconnected mechanisms that are responsible for temperature mediated life extension offer numerous targets for new aging interventions.

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